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Targeted gene replacement by homologous recombination in Drosophila stimulates production of second-site mutations

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Key words: Drosophila, BEAF, insulator, gene targeting, homologous recombination

Gene replacement by homologous recombination is a powerful technique for generating mutations in Drosophila. While using this technique for the *BEAF* gene, we encountered non-targeted lethal mutations on the target chromosome that complicated the analysis of the *BEAF* mutations until they were discovered and removed by meiotic recombination. Subsequent experiments indicated that the gene-targeting method leads to a modest but significant three-fold increase in the rate of production of non-targeted lethal mutations. It is important to be aware of this phenomenon when using this method.

Introduction

Gene targeting by homologous recombination allows specific mutations to be introduced into genes of interest. Since the development of this method in Drosophila in 2000, it has rapidly been adopted by researchers as an important part of their toolkit for generating mutations in flies.[1] To carry out gene targeting by the ends-in method in Drosophila, a donor construct carrying mutated DNA from the gene to be targeted is randomly inserted into the genome by P-element-mediated transformation (**Fig. 1**). Then, a site-specific recombinase (FLP) and a site-specific endonuclease (I-*Sce*I) are used to excise the mutated transgene as an extra-chromosomal DNA molecule that carries a double-stranded break (DSB). The presence of the DSB stimulates homologous recombination between the excised donor and the homologous chromosomal target locus.[1,2] The desired fate of an ends-in targeting molecule is integration into the gene of interest, producing a tandem duplication of the targeted gene with a marker gene between the copies. The target locus duplication can be reduced to a single copy by homologous recombination between the repeated sequences, which also deletes the marker gene. This event is stimulated by an I-*Cre*I-generated DSB between the repeats.[3]

While using this technique to generate mutations in the *BEAF* gene,[4] we obtained second-site lethal mutations at unknown locations on target chromosomes. One lethal second-site mutation was apparently introduced at the same time as the gene targeting event (the FLP, I-*Sce*I step), while the other was introduced during reduction of the gene duplication to a single copy (the I-*Cre*I step). These unexpected mutations complicated the analysis of the *BEAF* mutations until they were discovered and removed (**Fig. 2**). Because the two chromosomes we isolated that lacked a wild-type *BEAF* gene both had second-site lethal mutations, this raised the question of whether the method of isolating mutations by gene targeting stimulates the production of non-targeted mutations. This question is addressed in this report.

Results

Determining lethal second-site mutation rates. The protocol for gene targeting by homologous recombination[1-3] was followed to determine the rates of occurrence of lethal second-site mutations. We used an isogenized third chromosome marked with a P[w^+ *mus301*] P-element[5] as the reporter, which we refer to as *3 iso* (Materials and Methods). Five schemes were used, as illustrated in **Figure 3**. In all cases, *3 iso* males were crossed to appropriate females (F_0 cross) and the larvae were heat shocked for one hour at 37°C. In the two experimental strategies, the starting females had a mutant *BEAF* "donor" transgene (*mBF*) on the *X* chromosome and transgenes encoding either heat shock-inducible *FLP* and *I-SceI* transgenes or a heat shock-inducible *I-CreI* transgene. The *mBF* transgene has recognition sites for FLP, I-*Sce*I and I-*Cre*I, and was previously used to generate mutations in the *BEAF* gene on the second chromosome by homologous recombination.[6] For the negative control, the starting females lacked *mBF*, *FLP*, *I-SceI* and *I-CreI* transgenes. For the "no donor" controls, the starting females lacked the *mBF* transgene but had either the *FLP* and *I-SceI* transgenes or the *I-CreI* transgene. Single males were used in the F_1 cross to allow subsequent determination of the number of *3 iso* chromosomes that picked up a lethal mutation. Following the cross strategies outlined in **Figure 3**, the chromosomes with

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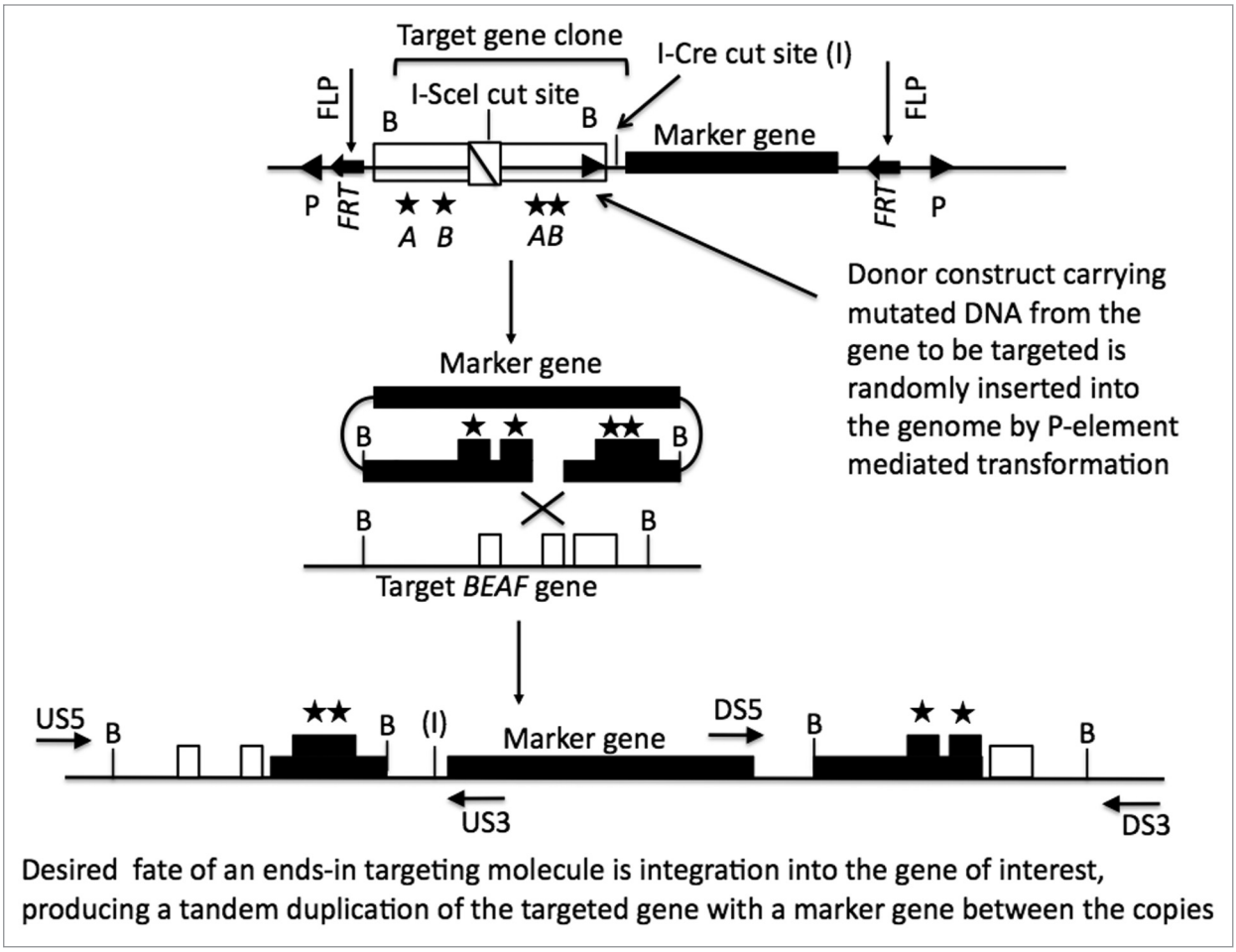

Figure 1. Shows the process of gene targeting event by homologous recombination using the ends-in method. A donor construct carrying mutated DNA from the gene to be targeted is randomly inserted into the genome by P-element-mediated transformation. Then, a site-specific recombinase (FLP) and a site-specific endonuclease (I-*Sce*I) are used to excise the mutated transgene as an extra-chromosomal DNA molecule that carries a double-stranded break (DSB). The presence of the DSB stimulates homologous recombination between the excised donor and the homologous chromosomal target locus. The desired fate of an ends-in targeting molecule is integration into the gene of interest, producing a tandem duplication of the targeted gene with a marker gene between the copies. (*A*, *B* & *AB* = represent the sites at which point mutations (*) are inserted into the donor construct, B = *Bgl*II restriction enzyme cut site, US5/US3 and DS5/DS3 = upstream and downstream PCR primers respectively).

the *mBF*, *FRT*, *I-SceI* and *I-CreI* transgenes were removed leaving flies with *3 iso* from a single male balanced over a *TM3* chromosome. These *3 iso*/*TM3* flies were self-crossed, and if all flies in the next generation had *TM3* then *3 iso* had picked up a lethal mutation.

Results of these crosses are presented in **Table 2 and Supplementary Tables A and B**. We found a spontaneous lethal mutation rate of 2.95% in the negative control. Others have reported spontaneous mutation rates of around 0.6%.[7,8] Our rate might be higher because of the strains we were using, and it might have been stimulated by the heat shock.[9] A chi-square analysis indicated that the lethal mutation rates in the *FLP*, *I-SceI* and the *I-CreI* "no donor" controls were not significantly higher. However, the roughly three-fold higher mutation rate for flies with the *mBF*, *FLP* and *I-SceI* transgenes was significant at a confidence level of nearly 95%, and the roughly 3.5-fold higher mutation rate for flies with the *mBF* and *I-CreI* transgenes was significant at a confidence level of nearly 99%. Perhaps activation of DNA repair pathways by the double-strand breaks present during these processes led to chromosomal instability at other locations.[10,11] It is also possible that the I-*Sce*I and I-*Cre*I endonucleases acting at suboptimal recognition sites in the genome contributed. Regardless of the mechanism involved, our results indicate that expressing these endonucleases in flies with perfect cut sites for these enzymes leads to a modest but significant increase in the rate at which non-targeted mutations arise.

In addition to the lethal mutations described above we also obtained unexpected recombination products (**Table 1**). This was discovered during the molecular characterization of these products.[6] Unexpected recombination products after gene targeting have been reported by other Drosophila researchers, such as during the generation of *Su(var)3-7* mutations[12] and *Nap1* mutations.[13] Additionally, insertions or deletions have been found after some gene targeting events, such as at the *yellow*,[1] *Su(var)3-7*,[12] and *pug* and *CG11305* genes.[3] Although the various products

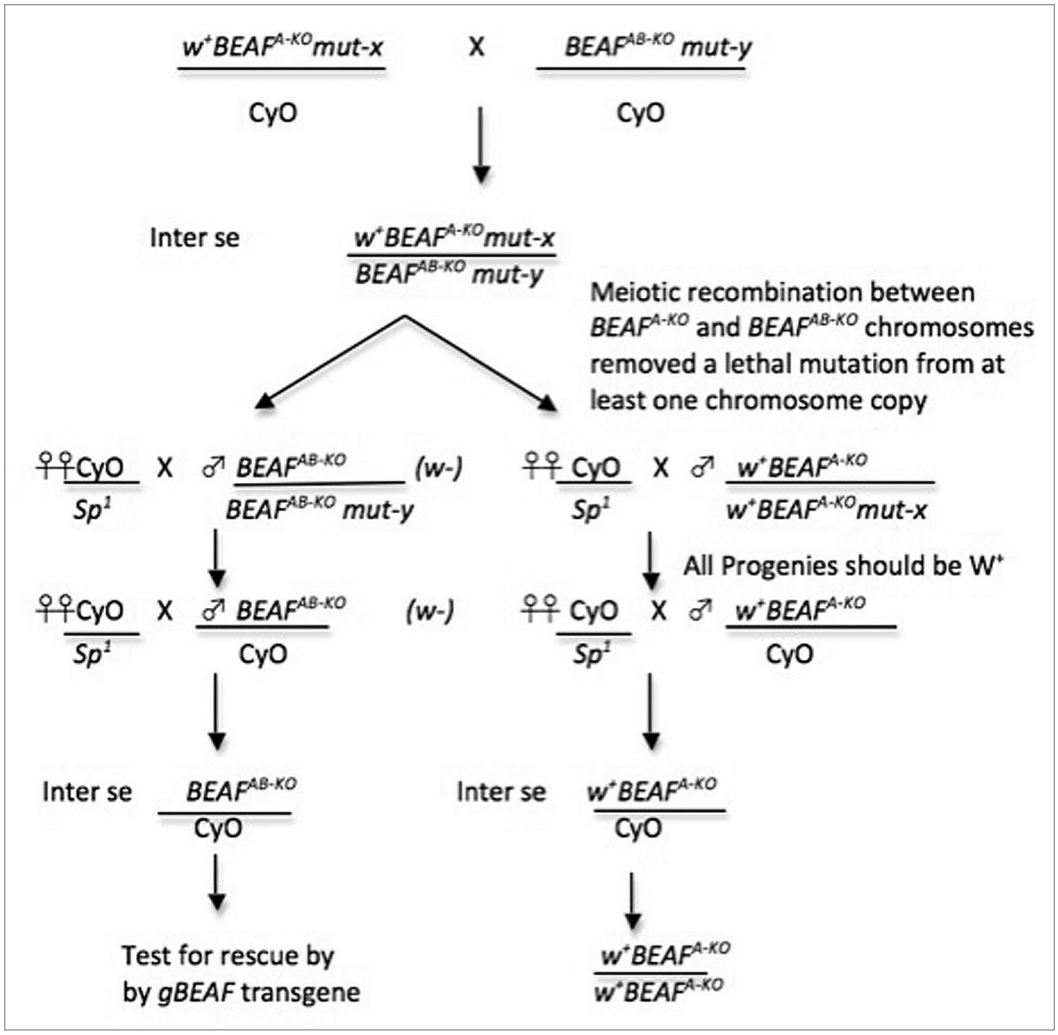

Figure 2. Genetic cross scheme for removing unwanted mutations from the *BEAF*$^{AB\text{-}KO}$ and *BEAF*$^{A\text{-}KO}$ chromosomes by meiotic recombination. Although both mutant *BEAF* chromosomes were recessive lethal, they were viable together. The chromosomes could only be homozygous if meiotic recombination removed a lethal mutation from at least one chromosome copy. The extra mutation on the *BEAF*$^{A\text{-}KO}$ chromosome (*mut-x*) was embryonic lethal. The extra mutation on the *BEAF*$^{AB\text{-}KO}$ chromosome (*mut-y*) was embryonic lethal together with the *BEAF* mutation, while animals died as pharate adults in the presence of a *BEAF* rescue transgene. Single males were crossed to *CyO*/*Sp*^{1} virgin females as indicated to isolate single mutant *BEAF* chromosomes for further characterization. It was difficult to determine if the *BEAF*$^{A\text{-}KO}$ chromosome was homozygous by eye color, so the second cross to *CyO*/*Sp*^{1} was only performed if all progeny of the first cross were w^{+}. Note that all experiments reported in Roy et al. (2007) were performed after removing the second-site mutations.

differ in detail, they are likely due to nonstandard events occurring during classical DSB repair.[3,13,14]

Evidence of occurrence of second-site mutations reported in several journals. Detecting and eliminating second-site mutations was essential for the accurate analysis our *BEAF* mutations. At least two other reports mention the presence of second-site mutations after generating mutations in Drosophila by homologous recombination,[13,15] suggesting the occurrence of these events is not uncommon. We conducted a survey of eight publications describing phenotypes associated with alleles generated by homologous recombination in Drosophila, and found that three did not mention experiments that would have determined whether non-targeted mutations affected observed phenotypes.[12,13,16-21] Four papers did not mention testing for rescue of mutant phenotypes by wild-type transgenes, and three of these four only reported experiments done with the mutant chromosome in a homozygous state. They did not combine mutant alleles with each other or with an appropriate chromosomal deficiency (which would keep second-site mutations heterozygous). Because of this plus our experience with this method, we feel it is important to raise awareness of the necessity to use care when analyzing mutations generated by gene targeting.

Discussion

Gene targeting by homologous recombination in Drosophila is a valuable tool. However, there is a distinct possibility that second-site mutations are often introduced into chromosomes

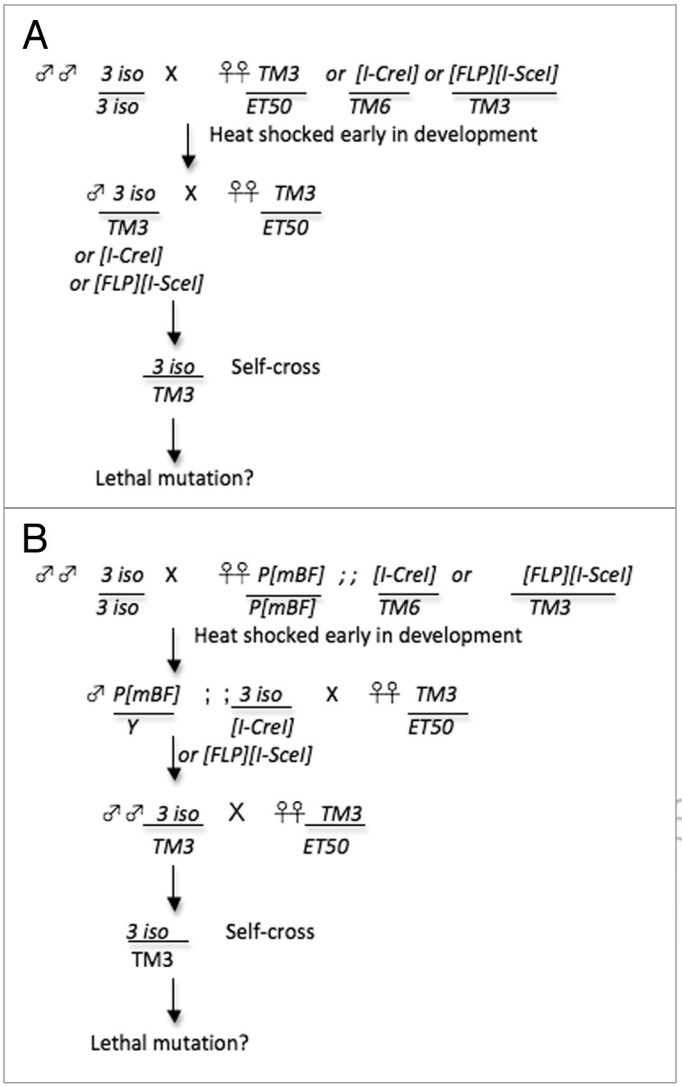

Figure 3. Genetic cross scheme for measuring spontaneous lethal mutation rates on the third chromosome. In the F_0 cross, males homozygous for an isogenized third chromosome marked with P[w^+ *mus301*] (termed *3 iso* here) were crossed to appropriate virgin females to result in the indicated genotypes. Larvae were heat shocked in a water bath at 37°C for 1 hour. This induces expression of the endonuclease transgenes, when present. F_1 males of the indicated genotypes were individually crossed to *TM3*/*ET50* female virgins (*ET50* is *Scm*^{ET50}). For the control crosses, resulting F_2 *3 iso*/*TM3* progeny were self-crossed to test for viability (A). The control crosses lacked the P[w^+ *mBF*] "donor" transposon. In addition, the negative control crosses lacked endonuclease transgenes, while the endonuclease control crosses did not. For the experimental crosses, F_2 *3 iso*/*TM3* males were again crossed to *TM3*/*ET50* female virgins to eliminate the *X* chromosome with the P[w^+ *mBF*] "donor" transposon, and the resulting F_3 *3 iso*/*TM3* progeny were self-crossed to test for viability (B). The P[w^+ *mBF*] transposon contains recognition sites for the FLP, I-*Sce*I and I-*Cre*I endonucleases.

when using this technique despite the expectation that only targeted mutagenesis will occur. Although this possibility is not addressed in many publications reporting use of this technique, detecting and eliminating second-site mutations was essential for the accurate analysis our *BEAF* mutations.[6] While the mechanism responsible for introducing the second-site mutations remains uncharacterized, our results and those of others highlight the need for care in working with mutations generated using homologous recombination.

Here in this paper we carried out the gene targeting method in experimental lines using a mutant *BEAF* transgene. Our goal was to measure the rate of production of lethal mutations on a non-targeted chromosome in the presence and absence of the I-*CreI* or I-*SceI FLP* transgenes, with and without a transgene containing recognition sites for these enzymes. Our results clearly indicate a significant increase in the rate of formation of non-targeted or second-site lethal mutations in the experimental lines.

As with other mutagenesis methods, well-established techniques such as backcrossing, mapping, complementation with a wild-type transgene, and the use of multiple independently derived alleles must be used to verify that observed phenotypes are attributable to the mutation of interest. Bearing this potential complication in mind, gene targeting is a powerful technique that allows the generation of mutant alleles that would otherwise be difficult to obtain.

Materials and Methods

Isogenizing the third chromosome. The third chromosome used in these crosses must be free of any lethal mutations. For this purpose it was isogenized. The third chromosome we selected is marked by a P[w^+*mus301*] transgene.[5] The w^+ confers orange eye color. Homozygous males of the P[w^+*mus301*]/P[w^+*mus301*] genotype were crossed with TM3/ET50 females. Male progeny of the genotype P[w^+*mus301*]/TM3 emerging from this cross were individually crossed to TM3/ET50 females again. Progeny flies of the genotype P[w^+*mus301*]/TM3 were then self-crossed, and flies homozygous for P[w^+*mus301*]/P[w^+*mus301*] (we refer as *3 iso*) from these vials were then self-crossed and maintained as a stable line.

Fly crosses. Five cross schemes were adopted. The first three crosses gave rise to the control lines and remaining two crosses generated the experimental lines.

Negative control line: This control line lacks the mutant *BEAF* transgene (P[w^+*mBF*]) on the X chromosome[6] and transgenes for any of the recombinases (I-*Cre*I or I-*Sce*I or FLP).[1,3] *3 iso* males were crossed with TM3/ET50 virgin females. The vials were emptied after 3 days and progeny larvae were heat shocked for 1 hr at 37°C in a water-bath. Adult P[w^+*mus301*]/TM3 males eclosing from these vials were then crossed individually with TM3/ET50 females. By this stage ~250 vials in 6 different sets were set up (**Suppl. Table A**). P[w^+*mus301*]/TM3 progeny emerging from these vials were self-crossed. In the next generation, vials giving rise to ~1/3rd of flies homozygous for P[w^+*mus301*]/P[w^+*mus301*] are considered non-lethal events, while vials with either none or very few (≤5% of total population) homozygotes are considered lethal or semi-lethal events respectively.

"No donor" controls (2 lines): These control lines lack the P[w^+*mBF*]. To establish these two lines, *3 iso* males were crossed to I-*CreI Sb*/TM6 or I-*SceI FLP*/TM3 females separately. Vials were emptied after 3 days and the progeny larvae were heat shocked as mentioned above to produce the FLP recombinase

Table 1. Results of homologous recombination

	Upstream *BEAF* gene				Downstream *BEAF* gene			
Fly line	**32A**	**32B**	**I-*Sce*I**	**Stop**	**32A**	**32B**	**I-*Sce*I**	**Stop**
Expected	WT	WT	-	M	M	M	-	WT
R1	M	WT	-	WT	M	WT	-	WT
R2	M	M	-	M	WT	WT	-	WT
R2*	M	M	-	M				
R3	WT	WT	-	WT	M	WT	-	WT
R4	WT	WT	-	WT	WT	WT	-	WT

32A: *32A* ATG start codon;[6] 32B: *32B* ATG start codon; I-*Sce*I: I-*Sce*I site introduced into the intron between the unique *32B* exon and the exon shared by *32A* and *32B*; Stop: conversion of the *Bam*HI site in the shared exon into two tandem stop codons; R: Recombinant *BEAF* fly line; WT: wild-type *BEAF* sequences; M: mutated *BEAF* sequences; -: no I-*Sce*I site. R2*: Fly stock derived from R2 by using I-*Cre*I to reduce the gene duplication to a single copy. There is a mini-*white* marker gene between the upstream and downstream copies of *BEAF*. Reduction to a single copy also eliminated the mini-*white* gene. Gene-specific PCR was used to amplify the genes, followed by restriction digestion analysis and sequencing.[6] The presence of M sequences where WT sequences were expected is unexpected. The presence of WT sequences where M sequences were expected is likely due to DNA repair occurring during branch migration during homologous recombination. These repair results are similar to those reported elsewhere.[3,12,13]

Table 2. Frequency of second site mutations

Genotype of heat-shocked male larvae	No. of crosses	Lethal	% Lethal	Chi-square	p
w; ; *3 iso*/TM3	237	7	2.95%		
w; ; *3 iso*/P[*hs-I-CreI*] *Sb*^{1}	105	3	2.85%	0.0528	0.8183
w; ; *3 iso*/P[*70-FLP*] [*70I-SceI*]	108	4	3.7%	0.0655	0.7980
P[*w*$^{+}$ *mBF*]; ; *3 iso*/P[*hs-I-CreI*] *Sb*^{1}	118	11	9.32%	6.433	0.0112
P[*w*$^{+}$ *mBF*]; ; *3 iso*/P[*70-FLP*] [*70I-SceI*]	114	8	7.01%	3.913	0.0479

See the legend to Figure 3 for details.

and site-specific endonucleases.[1-3] P[*w*$^{+}$*mus301*]/I-*CreI Sb* or P[*w*$^{+}$*mus301*]/I-*SceI FLP* males emerging from this cross were then individually mated with TM3/ET50 females. By this point ~150 vials for each control line were set up in 3 separate sets (**Suppl. Table A**). P[*w*$^{+}$*mus301*]/TM3 adults eclosing (eliminating I-*CreI Sb* and I-*SceI FLP* chromosomes) were then self-crossed and in the next generation the number of lethal vs. non-lethal events were calculated.

Experimental lines (2 lines): First P[*w*$^{+}$*mBF*]/P[*w*$^{+}$*mBF*]; I-*CreI Sb*/TM6 and P[*w*$^{+}$*mBF*]/P[*w*$^{+}$*mBF*]; I-*SceI FLP*/TM3 stable lines were established. *3 iso* males were crossed with females from each of the above two lines separately. Vials were emptied of flies after 3 days and they were heat shocked as mentioned above. Males of the P[*w*$^{+}$*mBF*]; P[*w*$^{+}$*mus301*]/I-*CreI Sb* or P[*w*$^{+}$*mBF*]; P[*w*$^{+}$*mus301*]/I-*SceI FLP* were isolated and crossed to TM3/ET50 females. Around ~150 vials in 3 sets (**Suppl. Table B**) were set up by this point. In the next step males of the genotype P[*w*$^{+}$*mus301*]/TM3 (getting rid of P[*w*$^{+}$*mBF*], I-*SceI FLP* and I-*CreI Sb*) were crossed with TM3/ET50 females. Progeny males and females of P[*w*$^{+}$*mus301*]/TM3 genotype were then self-crossed. Progeny from these crosses were scored to determine the number of lethal vs. non-lethal events.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/RoyFLY4-1-Sup.pdf

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